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Binding of small acid-soluble spore proteins from *Bacillus subtilis* changes the conformation of DNA from B to A

(CD/Fourier-transform infrared spectroscopy/DNA photochemistry/UV resistance)

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ABSTRACT **Small acid-soluble spore proteins (SASPs) appear 3–4 hr after the onset of sporulation in Gram-positive bacteria and constitute up to 20% of the protein of mature spores. Previous studies using *Bacillus subtilis* deletion mutants lacking SASP-α and -β have shown that such mutations abolish the elevated resistance of spores to UV radiation. Analyses using circular dichroism and Fourier-transform infrared spectroscopy now demonstrate that binding α/β-type SASPs to DNA *in vitro* causes a structural change in DNA, from the B to the A conformation. This may provide the basis whereby α/β-type SASPs confer increased spore UV resistance *in vivo*—by changing spore DNA conformation, they alter DNA photochemistry such that UV irradiation produces spore photoproduct instead of the more lethal cyclobutane-type thymine dimers.**

Among the dramatic changes accompanying sporulation in Gram-positive bacteria, a major increase in resistance to killing by UV radiation ranks high as a factor ensuring that the organism will survive dormancy (1). Detailed study of this phenomenon has shown that UV irradiation of spores produces no detectable cis–syn cyclobutane-type thymine dimers in DNA—in sharp contrast with the predominance of this lesion in other UV-irradiated *in vivo* systems. Instead, the DNA of UV-irradiated spores accumulates primarily 5-thyminyl-5,6-dihydrothymine ("spore photoproduct") (2, 3). Germinating spores contain a specific enzyme that efficiently repairs spore photoproduct (4), thereby ensuring a high probability of survival after exposure to UV.

It is clear that DNA conformation can significantly alter photoreactivity (5) and the type of UV-induced photoproducts (6). Thus, one simple explanation for the change in DNA photochemistry that occurs during sporulation invokes an alteration in DNA conformation. Indeed, to explain the altered photochemistry of spore DNA, it was suggested a number of years ago that DNA in bacterial spores is in the A conformation whose stereochemical constraints inhibit thymine dimer formation (7). However, the relationship between A-DNA and UV-induced formation of spore photoproduct has never been proven, and it has been suggested (6, 8) that hydration is a more important determinant of DNA photochemistry.

Although spores are significantly dehydrated relative to growing cells (9), that factor alone does not account for their UV resistance. Analysis of the properties of appropriate deletion mutants has shown that α/β-type small acid-soluble spore proteins (SASPs), which are associated with spore DNA *in vivo*, play an essential role in protecting spores against UV damage (10). These proteins, ranging in size from 60 to 73 amino acid residues (5–7 kDa), have been detected in spores of all species of Gram-positive bacteria tested and are coded for by a multi-gene family. The products of these genes (termed *ssp* genes) exhibit a remarkably high degree of conservation of their primary sequences throughout evolution (10), and any one of the α/β-type SASPs appears capable of conferring UV resistance on bacterial spores (11). By using several purified α/β-type SASPs, we have examined the effects of SASP on DNA conformation, and we report here that SASP binding to DNA *in vitro* induces a B → A conformation change. It seems likely that this phenomenon underlies the ability of SASP to protect spore DNA against thymine-dimer formation. Given their evolutionary antiquity (10), the SASPs also may have as-yet-undiscovered protein relatives that use the same strategy to protect cells against UV. The high intrinsic UV resistance of obligate anaerobic bacteria (12), for example, may reflect just such a system.

MATERIALS AND METHODS

Bacillus subtilis SASP-β and -γ were purified from spores of $\alpha^-\gamma^-$ and $\alpha^-\beta^-$ mutants (13, 14), respectively, essentially as described (15). These proteins were at least 85% pure, based on analysis by electrophoresis on SDS/polyacrylamide gels. The α/β-type SASP SspC came from the cloned *B. subtilis sspC* gene expressed in *Escherichia coli* and was purified as described (16). This protein gave only a single band on heavily loaded acid polyacrylamide electrophoresis gels, indicating a purity of >98% (16). Its amino acid composition agreed exactly with that predicted from the sequence of the gene (data not shown). The dry lyophilized SASPs were dissolved in Mes buffer (5 mM Mes/0.1 mM EDTA, pH 7.0) and any insoluble residue was removed by decantation or centrifugation. Protein concentrations were determined by absorbance measurements and/or colorimetric assay (17). The latter techniques were standardized by amino acid analyses using the compositions derived from the protein sequences.

Calf thymus DNA (Sigma type I) was dissolved in Mes buffer overnight at 4°C and sonicated before use. *E. coli* and *B. subtilis* DNAs were prepared by standard procedures including phenol extraction. Freedom from residual protein was demonstrated by UV absorbance measurements (A_{260}/A_{280} = 1.89–2.05) (18). These DNA samples showed the expected hyperchromicity when denatured at 100°C.

Circular dichroism (CD) spectra were taken on a Cary model 61 instrument at 25°C by using 1-mm and 10-mm pathlength cells. Noise levels typically were ±1–2 millidegrees at a scan rate of 10 nm/min. Samples displaying visible turbidity were rejected; the absence of significant ellipticity above 300 nm ruled out artifactual scattering.

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Abbreviations: SASP, small acid-soluble spore protein; FTIR, Fourier-transform infrared spectroscopy.

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Fourier-transform infrared (FTIR) spectra were obtained with a Perkin–Elmer model 1800 spectrometer interfaced to a Perkin–Elmer model 7500 computer. (Some measurements were also made on a Nicolet model MX-1 spectrometer. In parallel experiments the two spectrometers gave completely analogous results.) All measurements were done in the absorption-mode single-beam regime. Two hundred scans were collected, processed with weak Beer–Norton apodization, and Fourier-transformed to get spectra with nominal 2-cm^{-1} resolution. We used the deuterated triglycine sulfate (DTGS) detector and the CDS-3 operating software package. Solvent H_2O absorption was subtracted from DNA and SASP/DNA spectra by an interactive difference routine using the OBEY program. We used three criteria to establish correct solvent subtraction: (*i*) absence of the H_2O association band at 2130 cm^{-1} (neither DNA nor SASPs absorb at this frequency, so this line is a suitable internal standard for solvent subtraction) (19), (*ii*) presence of a flat baseline in the 2000- to 1000-cm^{-1} range, and (*iii*) absence of negative side lobes. Enhancement and derivatization (using the Savitsky–Golay function) of spectra were performed using the CDS-3 programs.

Samples for FTIR were prepared by mixing DNA (6 mM phosphate in DNA) and SASP solutions (6 mM NaCl/1 mM sodium phosphate, pH 7.0) and spreading them as a film on AgCl windows, which were exposed to room humidity to dry before placing them in the infrared cell. For work with films, the windows had a spacing of 2 mm and the hermetically sealed cell was connected from two ports by tubing to a chamber containing saturated solutions of appropriate salts and equilibrated for 24 hr to give the desired humidity (20). For solution spectra the solutions were enclosed between two CaF_2 plates with a Teflon spacer (25- or 50-μm pathlength). Background absorption in the region of interest for these experiments (1100 cm^{-1} to 1300 cm^{-1}) was minimal.

RESULTS

Fig. 1 shows the results of a typical CD titration experiment using *B. subtilis* SASP-β and *E. coli* DNA. Over the wavelength region shown, the protein makes no contribution to the CD spectrum (S.C.M. and C.H., unpublished observations). Comparison with reference spectra (21–24) reveals that the change which occurs upon adding SASP-β to the DNA solution is consistent with a progressive conformation change from B-DNA to A-DNA. In particular the blue-shifted wavelength of maximum ellipticity at the highest SASP concentration (264 nm) agrees closely with that observed in low-humidity films (21) and in ethanolic solutions (22–24). The ellipticity also increases in magnitude in the region above 250 nm, consistent with the well-known transition from the conservative CD spectrum of B-DNA to the nonconservative spectrum of A-DNA. (The CD spectrum of B-DNA has a lobe of positive ellipticity above 260 nm approximately equal in magnitude to the negative lobe in the range of 220–260 nm.) Because of the intense peptide CD bands centered around 225 nm, it is not possible accurately to measure the short-wavelength portion of the CD spectrum of the DNA complexed with SASP; thus we show only the longer-wavelength portion of the spectrum.

Fig. 2 shows the results of a similar titration experiment, this time with SspC, the α/β-type SASP produced by the *sspC* gene, binding to *B. subtilis* genomic DNA. Again there is a progressive shift in the spectrum from that characteristic of B-DNA to an A-DNA spectrum. Plotting the ellipticity at the wavelength of maximum change (260 nm) in this experiment as a function of the concentration of SspC at constant DNA concentration (Fig. 3) shows that the curve is sigmoid in shape and reaches saturation at an SspC/DNA weight ratio of about 3:1. A sigmoid binding curve for SspC–DNA interaction, possibly indicative of cooperative binding of SspC to DNA, has also been found when SspC–DNA interactions have been measured by other assays as has the saturation ratio of SspC/DNA = 3:1 (wt/wt) (16). When aliquots of these samples were electrophoresed on 1% agarose gel and stained with ethidium, there was a progressive retardation of the genomic DNA band with increasing protein/DNA ratio, paralleling the CD changes. This mobility-shift data (not shown) clearly demonstrate that the increase in A-like character of the CD spectrum corresponds to increased protein–DNA association.

Comparison of Figs. 1 and 2 shows that a 4:1 (wt/wt) ratio of SASP-β–DNA (Fig. 1, curve c) does not effect as great a change in the CD spectrum as a comparable SspC/DNA ratio (Fig. 2, curve f). Also, the maximum ellipticities reached at the highest protein/DNA ratios are not the same (Fig. 1, curve d, and Fig. 2, curve f). We attribute such variations to the different proteins and DNAs employed, the different concentrations used, and to the fact that the SASP-β was only about 85% pure.

By contrast with the results obtained for α/β-type SASPs, addition of *B. subtilis* SASP-γ to DNA solutions had no effect on their CD spectra, even at high protein/DNA ratios (data not shown). The lack of effect of SASP-γ on DNA conformation is consistent with the lack of a role for this protein in

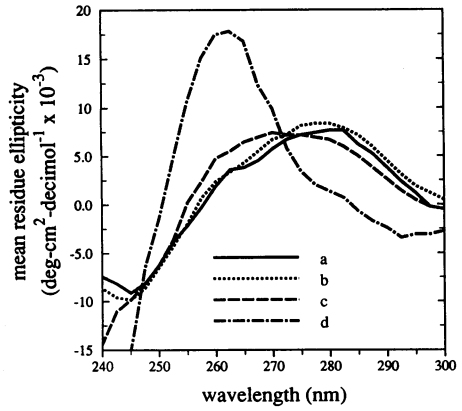

FIG. 1. CD spectral titration of *E. coli* DNA [92 μM (P)] with *B. subtilis* SASP-β. The SASP–DNA ratios are given in the square brackets. Curves: a, DNA alone [0]; b, DNA + protein (0.030 mg/ml) [1]; c, DNA + protein (0.12 mg/ml) [4]; d, DNA + protein (0.72 mg/ml) [24].

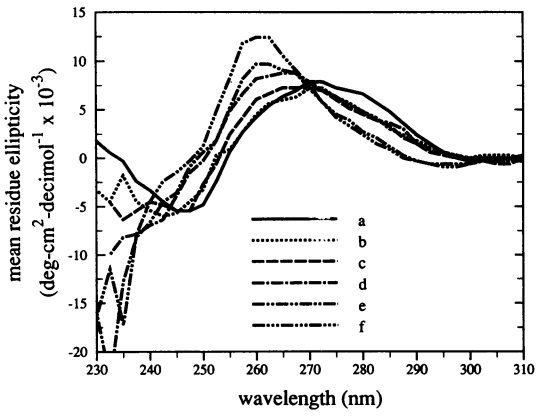

FIG. 2. CD spectral titration of *B. subtilis* DNA [0.14 mM (P)] with *B. subtilis* SspC. The SASP–DNA ratios are given in the square brackets. Curves: a, DNA alone [0]; b, DNA + protein (0.024 mg/ml) [0.5]; c, DNA + protein (0.048 mg/ml) [1]; d, DNA + protein (0.097 mg/ml) [2]; e, DNA + protein (0.15 mg/ml) [3]; f, DNA + protein (0.18 mg/ml) [4]; or DNA + protein (0.45 mg/ml) [10].

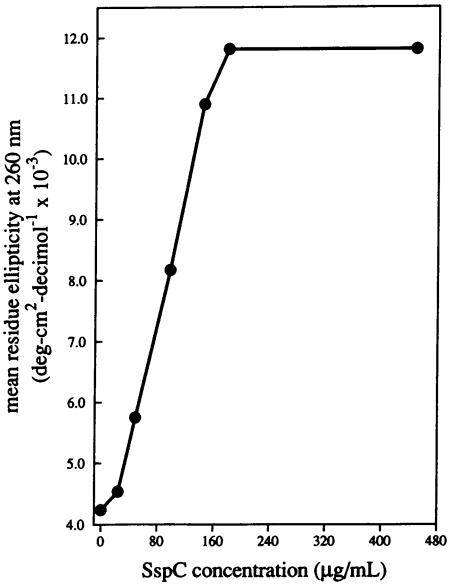

FIG. 3. Effect of SspC protein concentration on the mean residue ellipticity of *B. subtilis* DNA at 260 nm. Data taken from the experiment described in Fig. 2.

spore UV resistance (14) and the fact that SASP-γ is not appreciably bound to spore DNA *in vivo* (10).

FTIR spectroscopy provides an independent means of evaluating the conformational state of DNA complexed with α/β-type SASPs. Figs. 4 and 5 show the FTIR spectra of concentrated solutions containing calf thymus DNA or SspC–calf thymus DNA complexes in the range of 1150–1300 cm^{-1}, where the principal absorption band (at 1225 cm^{-1} in B-DNA) arises from the antisymmetric O–P–O phosphate stretching vibration (25). This band shifts from 1225 cm^{-1} in the film with DNA alone to 1246 cm^{-1} in the film containing the complex. Such behavior is characteristic of the B → A transition and in fact the dichroic ratios of the polarized components of this band have been used to estimate the geometrical details of the two conformations (25, 26). Since hydration effects alone also influence the position of this band, however (27), this result must be interpreted cautiously.

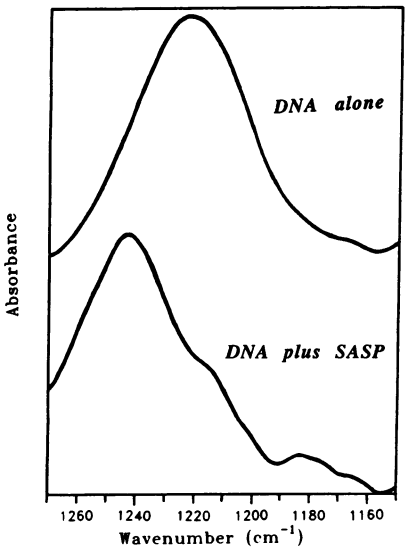

FIG. 4. Direct FTIR solution spectra of calf thymus DNA and the *B. subtilis* SspC–calf thymus DNA complex. Complex was prepared by mixing equal volumes of DNA (2 mg/ml) and SspC (2 mg/ml).

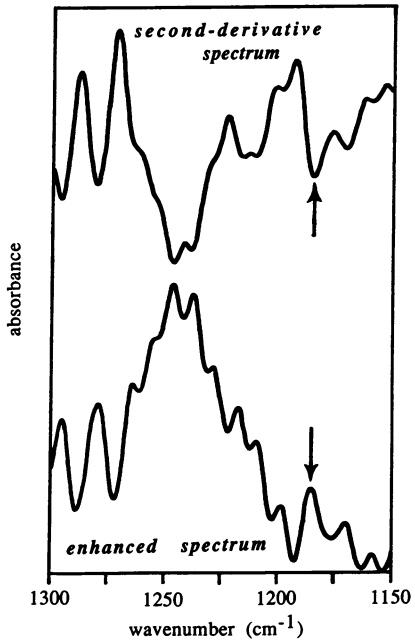

FIG. 5. FTIR derivative and enhanced spectra for the SspC–DNA sample shown in Fig. 4. Arrows mark the 1185-cm^{-1} band.

Another, unambiguous indication that the B → A transition has occurred comes from the appearance in the FTIR spectrum of the SASP–DNA complex of an absorption band at 1185 cm^{-1}, a specific marker for the A conformation (28). This band falls outside any protein absorption and consistently appeared in all SASP–DNA complexes examined under all humidity conditions (cf. Fig. 4). As shown in Fig. 5, the 1185-cm^{-1} band emerges very clearly in enhanced and derivative spectra. The SASP/DNA ratio in these experiments was 1:1 (wt/wt), (i.e., less than the saturation value obtained by other methods). Thus the band intensities shown in Figs. 4 and 5 do not necessarily represent the maximum attainable values.

Fig. 6 shows the effect of decreasing humidity on the band position for the antisymmetric phosphate stretch vibration. For control DNA films, the position only changes when the humidity drops significantly below 100%. For the SASP–

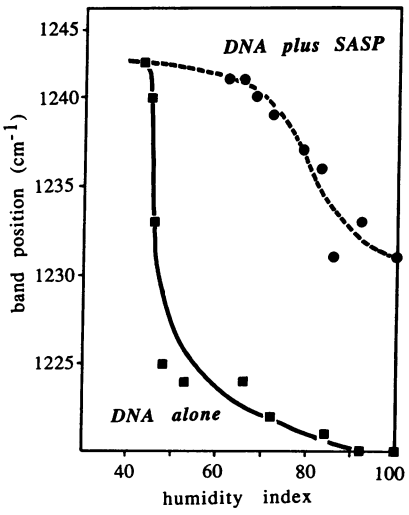

FIG. 6. Position of phosphate antisymmetric stretch as a function of humidity for films of calf thymus DNA alone and for the 1:1 (wt/wt) *B. subtilis* SspC–calf thymus DNA complex. Humidity index = $[(A_{3440})_x/(A_{3440})_{100}]100$, where x is the percent relative humidity at measurement and the subscript 100 indicates measurement at 100% relative humidity.

DNA complexes, however, the band position is already shifted at 100% relative humidity, and further decreases in the humidity lead to a complete shift to values characteristic of A-DNA much more rapidly than for films with DNA alone.

A similar pattern occurs with the 1185-cm^{-1} band (Fig. 7). In that case, however, we extended the observations to the lowest achievable humidity (≈5%). Although both the SASP–DNA complex and the DNA control give a maximum in the 1185-cm^{-1} band at about 70% relative humidity, the latter loses intensity much more rapidly with further decrease in the humidity. Such observations on bands attributable to DNA conformation (secondary structure) have been interpreted to mean that the molecule has become "disordered" (29).

DISCUSSION

CD changes have long been regarded as sensitive criteria for DNA conformations (21–24) and, though it may be difficult to assign fine structural details on the basis of CD spectra, the distinctions between the main families of secondary structure are unambiguous. The fact that interaction with α/β-type SASPs induces spectroscopic features characteristic of A-DNA with both eukaryotic and prokaryotic DNAs, including the homologous *B. subtilis* DNA, strongly supports the hypothesis that these proteins induce a B → A transition *in vitro*. By contrast, *B. subtilis* SASP-γ has no effect on the CD or FTIR spectra of DNA under all conditions tested—a result consistent with its biological role as an amino acid reservoir, but not a DNA-protective protein (14).

The FTIR results provide strong independent evidence for the SASP-induced B → A conformation change in DNA. Both the anti-symmetric stretch and the 1185-cm^{-1} bands behave exactly as one would predict for such a conformation change (25). Whereas hydration effects alone can influence the O–P–O stretching band (27), this complication does not affect the analysis of the 1185-cm^{-1} band, which originates principally from deoxyribose motions—and therefore responds to the differing sugar ring pucker in A-DNA compared to B-DNA. The ability to examine the complexes in films at various levels of humidity also adds important information. It is clear that dehydration can cause DNA to undergo the B → A conformation change, and it has been suggested that, since spores are significantly dehydrated, that factor alone can account for the alteration in their DNA photochemistry by formation of A-DNA (1). What our FTIR results clearly show, however, is that SASPs promote this conformation change such that it reaches completion with significantly less reduction in humidity than is required for the process with DNA alone.

Also of interest is the apparent protective effect SASPs have on the DNA under conditions where the nucleic acid

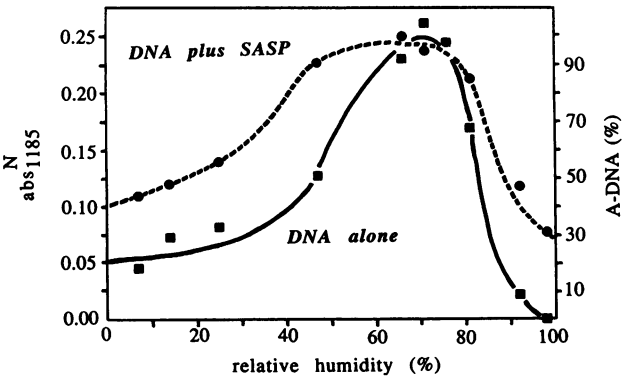

FIG. 7. Normalized intensities (N) for the 1185-cm^{-1} FTIR band of DNA and of 1:1 (wt/wt) SspC–DNA complexes as a function of humidity (28): $A^N_{1185} = A_{1185}/A_{1230}$.

alone would become "disordered" (Fig. 7). It seems clear that the SASP–DNA complex has greater stability toward disruption by drying than the pure DNA, and this may indicate an additional role for SASPs *in vivo*. In this connection we note that *Bacillus megaterium* SASPs have been shown to stabilize DNA against thermal denaturation (30).

The recent FTIR study of Dev and Walters (31) on complexes formed with a synthetic peptide and various DNAs provides an interesting comparison with the SASP–DNA system. Their peptide (29 amino acid residues) binds to DNA and the peptide undergoes significant conformation changes, but the bands assigned to DNA shift only slightly, possibly reflecting ionic or hydrogen-bonding interactions or subtle changes in helical conformation within the confines of an overall B-DNA structure. Thus there must be some special feature of the SASP structure that induces the B → A transition. Interestingly, the FTIR spectra of SASP–DNA complexes also clearly show conformational changes in the protein (unpublished observations).

As far as we know, this is the first report of a protein that alters the global secondary-structural parameters of DNA from one "family" to another upon binding after direct mixing at low ionic strength. SASPs bear some resemblance to the "histone-like" proteins of bacteria (such as *E. coli* HU protein), but these other bacterial proteins have only a minor effect on DNA conformation and, for the HU protein binding, the DNA CD is altered in the exactly opposite fashion to what we have observed with SASP (32). DNA complexed with HU appears to be overwound (i.e., altered in the direction of the D conformation). Since D-DNA remains within the B family of structures (22), the conformational alteration evoked by HU does not comprise as significant a change as that induced by SASP. Furthermore, examination of SASP–DNA complexes in the electron microscope does not show any beaded nucleosome-like structure (unpublished data), as has been reported for HU–DNA complexes (33).

Careful analysis of the infrared spectra of complexes formed between eukaryotic histones (or histone fragments) and DNA has likewise not demonstrated any major conformational change in the DNA but instead indicates a role for these proteins in stabilizing the B-DNA conformation (25, 34). This finding generally agrees with the extensive CD literature on the subject of histone–DNA interactions, except for the reports by Shih and Fasman (35, 36) that under certain conditions of complexation (step-gradient dialysis from 2 M NaCl to 0.15 M NaCl in the presence of 5 M urea, followed by removal of the urea and dialysis into 0.14 M NaF), calf thymus histone H4 over a limited range of protein–DNA ratios causes changes in the CD of DNA quite similar to the ones we have reported here. Under other conditions of complex formation, no CD changes of this type occurred, and cleavage of the H4 protein at its single methionine residue by cyanogen bromide abolished the phenomenon (37). Because these histone–DNA complexes displayed features—in particular significant optical density above 300 nm due to light scattering (36)—which suggest aggregation, the observed CD spectral changes may well have stemmed in large measure from liquid-crystal-like effects, as seen in ψ-DNA systems (38). Thus, although it is possible that under restricted conditions histone H4 has a similar effect on DNA conformation as do SASPs, the evidence remains somewhat tenuous. For histone H2B, Adler *et al.* (39) have reported related CD changes that, however, appear entirely interpretable in terms of aggregation/liquid-crystal-like effects. It is of interest in this regard that CD studies of complexes formed between DNA and polypeptide models for arginine-rich histones show no enhancement of the 275-nm band (in the direction of an A-like spectrum) but rather lead to a diminution of this band reminiscent of C-DNA (40).

A number of CD studies have examined the effects of gene regulatory protein binding to DNA. Nonspecific binding of *E. coli lac* repressor to DNA (41) and to synthetic polydeoxynucleotides (42) causes an increase in the 275-nm DNA CD band comparable to that we have observed with SASPs, but the ellipticity maximum does not shift below 270 nm. Thus the conformational change induced in nonoperator DNA by *lac* repressor—and by its "headpiece" (43)—cannot constitute a true B → A transition (41). Similar CD changes occur when the tet repressor binds *tet* operator DNA (tet repressor, however, has no effect on the CD spectrum of nonoperator DNA) (44). By contrast, specific *lac* repressor–operator binding leads to a distinctly different CD spectral change (45), closely paralleled by that of the *gal* repressor/operator-specific complex (46). In these cases a smaller increase in the ellipticity accompanies a shift of the maximum to slightly longer wavelength. A third type of behavior occurs with *cro* repressor binding to OR3 operator DNA (47) and cAMP receptor protein binding to DNA non-specifically (48). In both of these cases the 275-nm CD band diminishes in intensity and shifts to longer wavelength.

Several recent studies provide supporting evidence for our conclusion that SASP binding to DNA induces a B → A conformation change that underlies the UV resistance of spores. Nicholson and Setlow (49) have shown that incorporation of plasmids into spores results in a 50% increase in negative supercoiling of the plasmid DNA. This increase develops synchronously with the appearance of SASPs during sporulation, and spores formed from mutant cells that lack SASP-α and SASP-β give rise to plasmids with significantly less supercoiling. If SASP binding causes a B → A transition, it will unwind the helical pitch approximately from 10.4 to 11 base pairs per turn (50). In a covalently closed circular plasmid, conservation of the linking number requires that an increase in (positive) supercoiling occur to compensate for such helix unwinding. The combined actions of DNA gyrase and topoisomerase I, however, maintain a constant extent of net negative supercoiling *in vivo*, with the result that plasmids that have bound SASPs show a substantial increase in negative supercoils (as measured after phenol extraction and reversion to the B conformation). Nicholson *et al.* (16) have further demonstrated this effect *in vitro* by binding SspC protein to covalently closed relaxed plasmids in the presence of topoisomerase I. After removal of the proteins, the plasmids were negatively supercoiled to an extent consistent with the twist change predicted by the B → A transition. Finally, the same authors have shown that complexation of DNA with purified SspC *in vitro* alters the nucleic acid photochemistry such that the yield of cyclobutane-type pyrimidine dimers drops dramatically whereas that of spore photoproduct correspondingly rises (W. L. Nicholson, B. Setlow, and P.S., unpublished data).

Examination of available data bases has revealed few clear-cut relationships between the homologous SASP amino acid sequences and any other known proteins (unpublished work). With the exception of their distant connection with the HU proteins, SASPs appear to be unique in structure as well as in their effects on DNA conformation. It should prove interesting to elucidate the structural details of SASP–DNA complexes. Fruitful comparisons among the 23 sequenced SASPs should facilitate the identification of their DNA-binding motif as should site-directed mutagenesis of the cloned *ssp* genes. Further work on their relationship to other DNA-binding proteins offers the possibility of casting light on evolutionary developments during the pre-Phanerozoic era (3.5×10^9–0.6×10^9 years before present) when living cells confronted much more intense UV radiation at the earth's surface due to the absence of a stratospheric ozone layer (12).

This work is dedicated to Paul Doty on the occasion of his 70th birthday. We thank Mike Connors for initiating this collaborative project and for helpful discussions, and Mary Walsh for use of the Cary model 61 spectropolarimeter. This work was supported by grants from the National Institutes of Health (GM19698), the Army Research Office, and the Graduate School of Boston University (881-CH), and by a fellowship from the Icelandic Science Foundation.

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